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PERSPECTIVES IN BASIC SCIENCE

Importance of quantitative genetic variations in the etiology of hypertension

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Importance of quantitative genetic variations in the etiology of hypertension.

Recent progress has been remarkable in identifying mutations which cause diseases (mostly uncommon) that are inherited simply. Unfortunately, the common diseases of humankind with a strong genetic component, such as those affecting cardiovascular function, have proved less tractable. Their etiology is complex with substantial environmental components and strong indications that multiple genes are implicated. In this article, we consider the genetic etiology of essential hypertension. After presenting the distribution of blood pressures in the population, we propose the hypothesis that essential hypertension is the consequence of different combinations of genetic variations that are individually of little consequence. The candidate gene approach to finding relevant genes is exemplified by studies that identified potentially causative variations associated with quantitative differences in the expression of the angiotensinogen gene (*AGT*). Experiments to test causation directly are possible in mice, and we describe their use to establish that blood pressures are indeed altered by genetic changes in AGT expression. Tests of differences in expression of the genes coding for the angiotensin-converting enzyme (ACE) and for the natriuretic peptide receptor A are also considered, and we provide a tabulation of all comparable experiments in mice. Computer simulations are presented that resolve the paradoxical finding that while ACE inhibitors are effective, genetic variations in the expression of the *ACE* gene do not affect blood pressure. We emphasize the usefulness of studying animals heterozygous for an inactivating mutation and a wild-type allele, and briefly discuss a way of establishing causative links between complex phenotypes and single nucleotide polymorphisms.

Our intention in this article is to present the logical background that underlies studies aimed at understanding how quantitative genetic variations influence blood pressure. The starting point in this type of work is illustrated in Figure 1, which is a reproduction from the progress report of the Hypertension Detection and Follow-up Program (HDFP) Cooperative Group of a compilation of diastolic blood pressures measured mostly at home in close to 160,000 persons aged 30 to 69 years from 14 communities [1]. There were some deliberate biases in the choice of communities for inclusion in this study, but the data speak for themselves: Blood pressures are distributed in the population in a continuous fashion with the exact incidence of "hypertension" being to a substantial degree a matter of definition. Nevertheless, there is general agreement that, as summarized by Pickering [2], "*the relationship between arterial pressure and mortality is quantitative; the higher the pressure the worse the prognosis.*" However, it is also important to note the clear emphasis of the HDFP report that "*milder hypertension . . . bears the major burden of excess deaths attributable to elevated blood pressure.*" Assessing the cost versus benefit ratio of treating mild hypertensive individuals is not a simple task, but there is little doubt that a better understanding of the factors underlying the incidence of the condition can be used to improve this ratio. The distribution illustrated in Figure 1 is bound to include individuals who have conditions already known to cause hypertension, such as renal arterial stenosis or chronic renal failure, but the majority of individuals who fall within the cross-hatched areas have high blood pressures with no obvious cause—the definition of essential hypertension.

Determining the relative contribution of genetic and environmental factors to the incidence of essential hypertension has not been easy. However, family studies, such as those involving identical and nonidentical twins raised in the same or in different environments, are consistent in indicating, as Ward summarized, "*that genetic factors are primarily responsible for the observed familial aggregation of blood pressure within populations. Overall, it appears that approximately 60-70% of familial aggregation is ultimately due to genetic background, with cultural factors being responsible for the remainder*" [3].

Key words: blood pressure, essential hypertension, genetic etiology, candidate gene, computerized gene simulation, ACE inhibitors, angiotensinogen.

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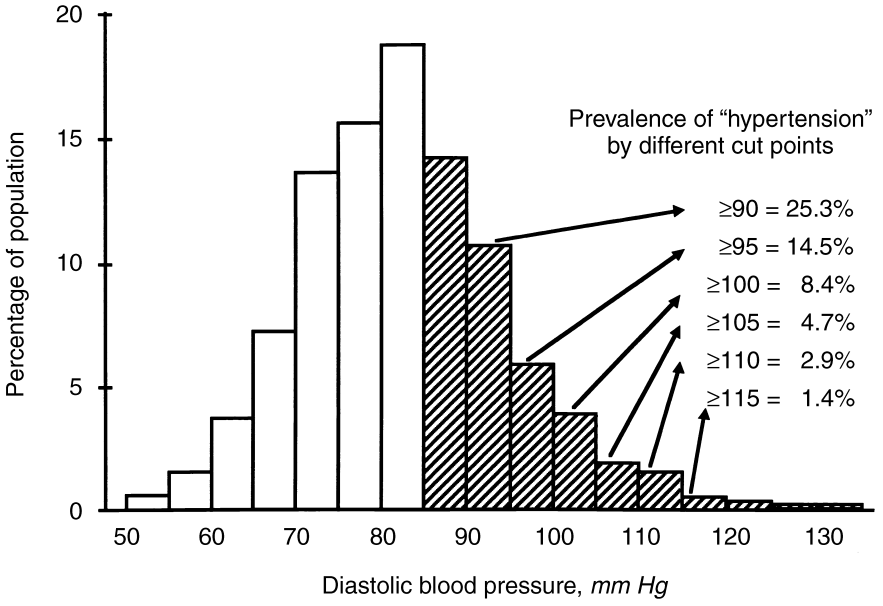

Fig. 1. Frequency distribution of diastolic blood pressures in 158,906 persons, 30 to 69 years of age. Redrawn with permission from the progress report of the Hypertension Detection and Follow-Up Program [1].

In other words, one can transmit beneficial or harmful "cultural factors" to one's offspring as well as genes.

The simplest patterns of genetic transmission are those that in honor of their discoverer we now refer to as Mendelian. Huntington's chorea, for example, is inherited in a dominant Mendelian fashion, a defective gene received from either parent being sufficient to cause the disease, while cystic fibrosis is inherited as a Mendelian recessive condition: Affected individuals must inherit a defective gene from each of their parents. Richard Lifton, Bernard Rossier, and their associates have pioneered efforts to uncover families in which Mendelian patterns of inheritance are important in the control of blood pressure. Glucocorticoid-remediable aldosteronism (GRA) is an example of a dominant form of hypertension caused by a genetic fusion of the regulatory sequences of 11β-hydroxylase with the coding sequence of aldosterone synthase [4], while mutations in the renal chloride channel CLCNKB [5] account for one form of Bartter's syndrome [6], a recessively inherited form of hypokalemic alkalosis with salt wastage and low blood pressure. However, these simply inherited Mendelian forms of hypertension or hypotension are for the most part rare, as pointed out by Lifton in a 1996 summary: "*mutations in at least 10 genes have been shown to alter blood pressure; most* . . . [in fact all but one, to which this article will return] . . . *are rare mutations*" [7].

A HYPOTHESIS

What, then, is the genetic basis for the common forms of essential hypertension? Our hypothesis, to which many geneticists would probably also subscribe, is that essential hypertension is the consequence of an unfortunate combination of genetic variations that are not necessarily the same in all of those affected and that individually may have only a modest effect on blood pressure. Indeed, the distribution of blood pressures in most of the general population could well be just one particular facet of the normal spectrum of human variability. Nevertheless, it is important to recollect that minor genetic differences, which may have been essentially neutral when they first arose, may have noticeable consequences in our modern society where individuals live well past their reproductive period. Genetic variations that have no deleterious effects in younger persons may prove harmful in an older population.

CANDIDATE GENES

A powerful method of uncovering genes of importance in determining a multifactorial trait is to first identify candidate genes by taking advantage of previous investigations that have defined physiological variables and biochemical systems affecting the trait. One such system, in the case of blood pressure, is the renin-angiotensin system. For a variety of reasons, including the fact that angiotensinogen (AGT) is at the head of this system, Jeunemaitre et al chose the *AGT* gene as a candidate [8]. Going from an initial selection of a candidate gene to proof that it is in fact important in determining the inheritance of hypertension can be accomplished by demonstrating three things: (*1*) that the gene has variants in human populations, (*2*) that the variants are inherited in families along with (that is, cosegregate with) the hypertensive trait, and (*3*) that the variants cause differences in blood pressure.

As a first step toward this end, these investigators took advantage of a highly polymorphic simple sequence, $(GT)_n$, known to occur close to the *AGT* gene [9] and found that the hypertensive siblings in families with at least two affected siblings inherited the same simple sequence variant more often than was expected by chance. This demonstration of cosegregation of simple sequence variants near the *AGT* gene with the hypertensive phenotype was sufficient to warrant looking for a possible causative difference within the *AGT* gene itself. The investigators therefore sequenced the coding exons and a short 5′ noncoding region of the *AGT* genes from some of their more hypertensive index cases and identified 15 distinct molecular variants. Comparing the frequencies of the variants in the hypertensive index cases with those in control normotensive siblings showed a significantly higher frequency in the hypertensives compared with the normotensives of one variant (threonine at position 235 instead of its alternative, methionine). An additional important observation was a significantly higher plasma concentration of AGT in the 235T-bearing hypertensive individuals, particularly women, than in individuals with the 235M allele.

These findings [genetic linkage of hypertension with the $(GT)_n$ sequence, the identification of molecular variants of the *AGT* gene, differences in their frequencies in hypertensive cases and controls, and a significant association of plasma AGT concentrations with *AGT* genotype] led the authors to hypothesize in suitably cautious terms that "*some molecular variants of angiotensinogen, such as those identified or tagged by the variant at residue 235, [may] lead to increased plasma or tissue angiotensinogen. . . . This could lead to a slight overreactivity of the renin-angiotensin system.*" However, proof that either the amino acid substitution and/or the difference in plasma AGT concentration caused changes in blood pressure was lacking.

GENE TARGETING

Genetic studies in the mouse are, however, very effective at testing causation. By using gene targeting [10], one can alter a gene in a predetermined manner and ask whether the imposed difference causes a change in the variable in question [11, 12]. Accordingly, our first plan was to change the amino acid at position 235 in mouse AGT from methionine to threonine. The experiment was short lived because mouse AGT does not have methionine at this position [13]. Indeed, this residue is poorly conserved in different species, suggesting that the specific amino acid at position 235 is relatively unimportant. Our second plan was therefore to alter the *AGT* gene in such a way that its level of expression would change. Theoretically, this should be possible by changing one or more nucleotides in the promoter of the gene. In practice, current knowledge of promoters is inadequate to allow an a priori decision of what changes to make in the sequence of a previously untested promoter to achieve a desired change. We therefore chose an alternative strategy to alter gene expression that is both universal in its applicability and predictable in its degree.

GENE TITRATION

The strategy, which we call "gene titration," is based on observations first made over 30 years ago by Harris, who summarized his findings with the statement: "*Where . . . [a protein] is completely . . . absent in the affected homozygotes, the values found in the heterozygotes are usually about 50% of those found in normal homozygotes. Thus there often seems to be a simple gene dosage relationship*" [14]. This means that to obtain a half normal level of expression in a mouse one can use the heterozygotes from a conventional gene knockout experiment, since they have only one functional gene in place of the usual two. The converse—how to obtain an increase in gene expression—is similarly presaged in the genetic literature by Epstein's compilation of data from mice and humans who have three copies of a normal gene, typically as a result of a chromosomal trisomy: "*When gene products of 37 human and 7 mouse loci were examined . . . the mean activities or concentrations in trisomic cells were 1.61 ± 0.25 (SD) and 1.55 ± 0.10 times normal (diploid) levels for the human and mouse*" [15]. How to replicate this situation deliberately is a little less obvious, but when targeting DNA is introduced in the form of a cut circle, the region of homology that is identical in the exogenous DNA and in the target gene ends up being duplicated. These two gene targeting techniques—gene disruption and gene duplication—are simply related to each other topologically [16]. The same DNA sequences can be used to obtain disruption of a gene or duplication of the same gene depending on how the sequences are assembled into a targeting construct (Fig. 2) [17]. An additional genetic trick is required when the gene to be duplicated is longer than can be accommodated into a practical targeting construct (as is usually the case). Yeast geneticists have known for some time that when sequences participating in homologous recombination are incomplete, gap repair occurs during the recombination and supplies the missing portion by using the target gene as a template [18]. This same phenomenon occurs in mammalian cells during gene targeting [19]. Consequently, by using gene targeting in the two modes, we were able to generate a series of animals in which the number of copies of the *Agt* gene varies systematically [20].

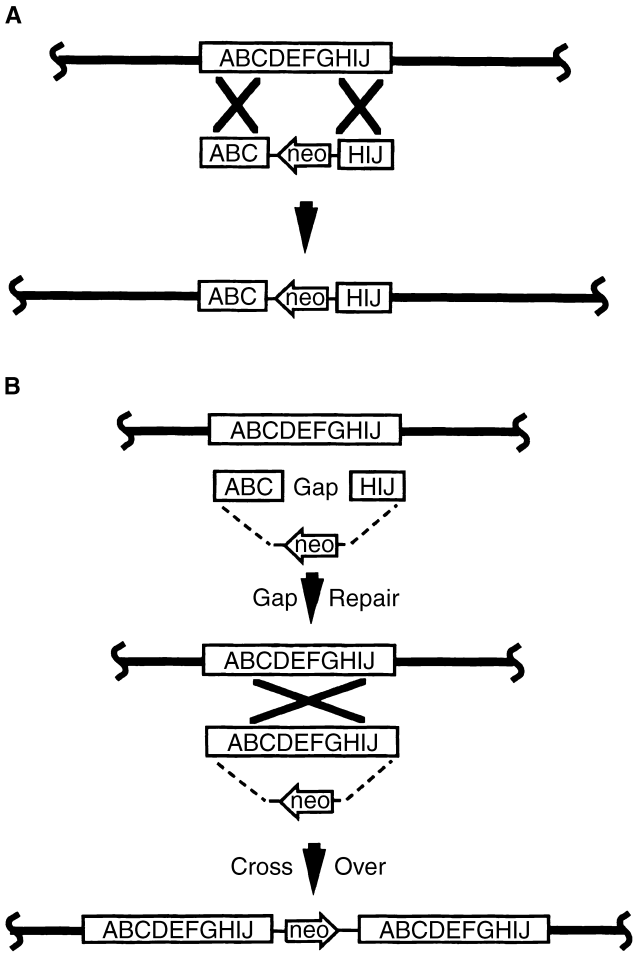

Fig. 2. Two modes of gene targeting: A, gene disruption and partial gene deletion; B, gene duplication. The top lines in the two parts of the figure represent the target gene, ABCDEFGHIJ; the next lower lines, the targeting constructs; the bottom lines, the modified genes. The selectable marker gene is neo. The dashed lines have no length. "Gap" indicates sequences missing in targeting construct B; they are repaired during the recombination. Regions where homologous crossing over occurs are shown by crosses. Note the different topological arrangements of the same DNA fragments in the two targeting constructs.

THE *AGT* GENE

Figure 3A shows that the steady-state concentration of AGT in the plasma of the resulting animals increases progressively with gene copy number and includes the range observed by Jeunemaitre et al in their hypertensive patients (~120% of that in the normotensive siblings) [8]. We measured the blood pressure of the animals and found, as illustrated in Figure 3B, that the blood pressures of the one-copy through three-copy mice also increased progressively with the *Agt* gene copy number. A firm conclusion can be drawn from these experiments, namely that genetically determined changes in the level of expression of the *Agt* gene directly cause changes in the blood pressure of mice, and that these changes are observable in animals that are otherwise wild type and have all their normal homeostatic mechanisms intact.

The significance of this conclusion with respect to humans is that it establishes in an animal model that modest genetically determined differences in the *Agt* gene that increase plasma AGT level by around 20% are sufficient to alter blood pressure, albeit by only a few mm Hg. The experiments should not be interpreted as suggesting that gene disruptions and gene duplications (both of which occur in humans, but are rare) contribute appreciably to the etiology of essential hypertension. Rather, the point is that small genetically determined differences in AGT expression, regardless of how determined at the DNA level, will affect blood pressure. It is therefore of considerable interest that more recent work by Inoue et al closes the circle in this particular argument, by demonstrating two things: (*1*) that the 235 polymorphism in the *AGT* gene is in tight linkage disequilibrium with an additional, single nucleotide polymorphic difference at position −6 in the promoter of the gene (G at −6 occurs almost exclusively with M at 235, while A at −6 occurs with T at 235), and (*2*) that the promoter with A at −6 is stronger than the promoter with G at −6 [21]. Thus, there is a single nucleotide polymorphic difference in the promoter of the T235 allele that can account for the higher expression of the T235 allele and so, by inference from the mouse data, for an increase in blood pressure. Published accounts of the effects on blood pressure of the *AGT* gene polymorphism in humans are in general agreement that modestly higher blood pressures accompany the A-6 and T235 variant [22].

HOMEOSTASIS

A complicating feature in the study of the genetics of hypertension is the need for complex organisms to maintain near constancy of their internal environments. This homeostasis is maintained by the operation of many different but often interacting systems of considerable sophistication that permit desirable physiological changes in biological variables, but that also act homeostatically if external factors cause undesirable changes in the variables. Such homeostatic adjustments in response to changes in external factors have been studied extensively in the past. However, little attention has been given to a categorical different set of homeostatic adjustments that must be made. These are the adjustments needed to accommodate the endogenous genetic differences that distinguish one individual from another. Homeostatic adjustments of this category occur in our mouse experiments and illustrate very well the difficulties that face investigators wishing to determine what genetic factors affect blood pressure. A clue to the extent of this category of homeostasis can be seen in the steady-state plasma level of AGT in the mice with only one functional copy of the *Agt* gene instead of the two copies present in the wild type; the level in the one-copy animals is only 35% of normal, which is signifi-

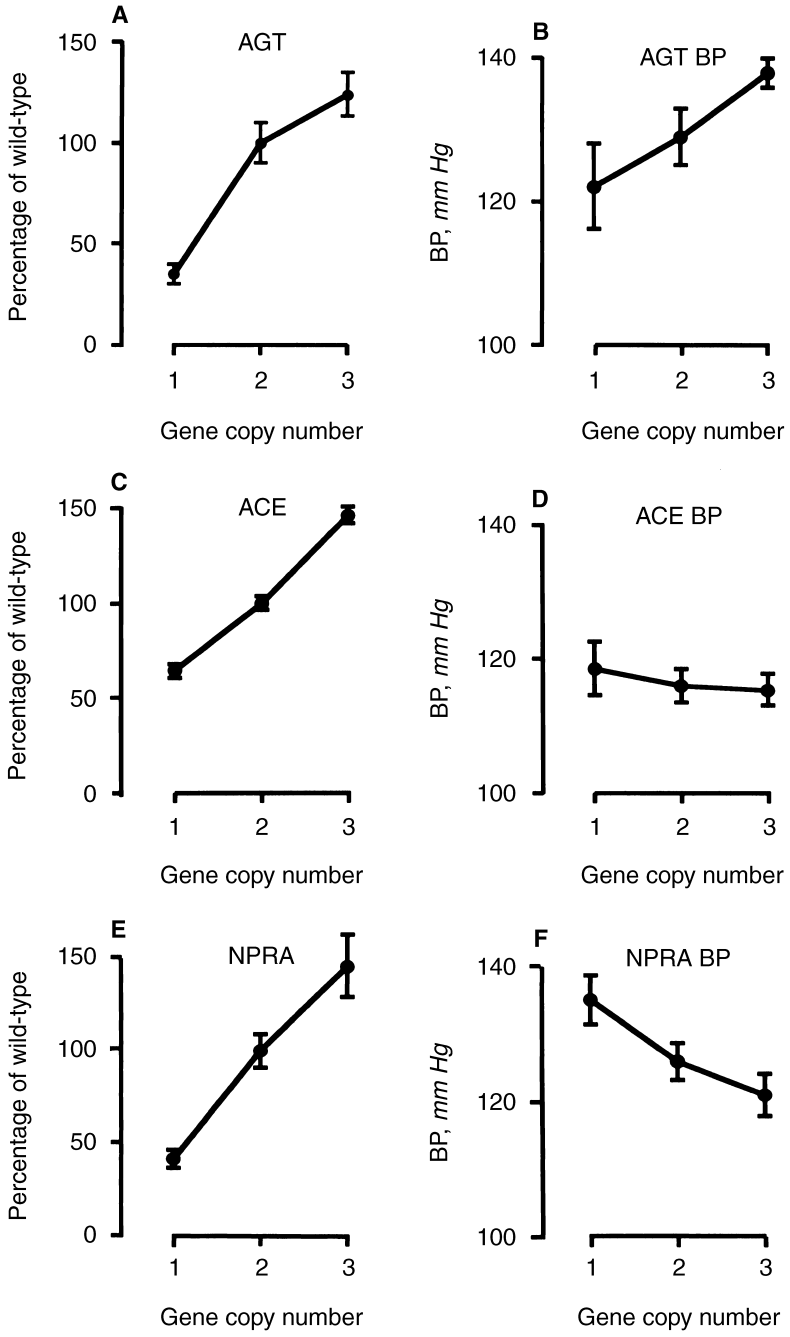

Fig. 3. Gene titration experiments in which the number of copies of three genes are varied and their effects on the amounts of encoded protein product as percentage of wild type (left panels) and on blood pressure (right panels) are determined. (*A*) Plasma angiotensinogen (AGT). (*B*) Blood pressures in *AGT* mice. (*C*) Serum angiotensin-converting enzyme (ACE) activities. (*D*) Blood pressures in *ACE* mice. (*E*) Guanylate cyclase activity of natriuretic peptide receptor A (NPRA) in the lung in the presence of excess atrial natriuretic peptide. (*F*) Blood pressures in *NPRA* mice. The primary sources of data are references [19, 22, 28].

cantly less than the expected 50% level. The cause of this low steady-state plasma AGT level proves to be a homeostatic increase in the number of cells producing renin in the kidney so that plasma renin levels are almost 250% normal [23]. However, despite this homeostatic increase in renin, the final result is a steady-state concentration of angiotensin II (Ang II) in the *Agt* one-copy mice still significantly less than normal, as are their blood pressures. Thus, in this instance, homeostatic changes reduce but do not abolish the effect on blood pressure of the genetically induced change in *Agt* gene expression.

Some additional comments are required before closing this discussion on homeostatic compensations induced by allelic differences. The data accumulated by Epstein, to which we referred initially [15], and our own experience with heterozygotes for disruption of many genes are in agreement with Epstein's summary statement that "*control mechanisms to regulate the final concentration of the gene product at some fixed level do not exist.*" [24] In other words, automatic up- or down-regulation of a gene to compensate for any genetic disturbance in its own expression or in that of its allelic partner does not occur. This does

not mean that compensations by specialized homeostatic systems have not been evolved, such as the changes in renin production that we have discussed or repression of the expression of cholesterol biosynthetic enzymes by intake of dietary cholesterol [25], but it does mean that these homeostatic compensations are not an automatic property of genes. A significant corollary of this conclusion is that the quantitative effects of allelic variants are strictly and algebraically additive so that, for example, if a heterozygote inherits one allele causing 120% average expression, with a second causing 70%, the net expression will be 95%.

THE *ACE* GENE

A second gene in the renin-angiotensin system that obviously merits attention as a candidate for affecting blood pressure is that coding for the angiotensin-converting enzyme (ACE). In addition to the enzyme being a key element in the system, its gene is a candidate because ACE inhibitors are such effective drugs for the treatment of hypertension and because in humans a common polymorphism in the gene is associated with a quantitative difference in the level of ACE in plasma [26]. Individuals homozygous for the *I* allele (gene frequency 0.4) have plasma ACE concentrations of 300 μg/L; individuals homozygous for the *D* allele (gene frequency 0.6) have 500 μg/L. Heterozygotes have 400 μg/L. Accordingly, we carried out a gene titration experiment with the *Ace* gene [27]. Figure 3C shows that plasma ACE activities in the resulting mice vary progressively, with the activities of the one- and three-copy animals ranging from approximately 65 to 150% of wild type. Surprisingly, however, as shown in Figure 3D, the blood pressures of the same animals are not affected by this nearly threefold change in plasma ACE activities. This result is paradoxical when one considers that ACE inhibitors lower blood pressure in mice [28] as they do in humans.

A possible explanation of the lack of effect of gene copy number on blood pressure might be that homeostatic compensations are sufficient to overcome the changes in the circulating ACE. However, the *Ace* one-copy mice have the expected half normal levels of ACE mRNA in their lungs (a major site of ACE synthesis) so that there is no evidence of any homeostatic compensation in the expression of the remaining normal *Ace* gene in these animals (our unpublished observations). A small but significant increase in kidney renin mRNA, of the order of 30%, does occur in the one-copy animals, but this seems unlikely to account for their normal blood pressures, since it is only about one third of the increase in kidney renin seen in the *Agt* one-copy animals and they still have below normal blood pressures.

COMPUTER SIMULATIONS

We therefore explored the possibility that the absence of differences between the blood pressures in the mice with one through three copies of the *Ace* gene is due to a different phenomenon, namely, the intermediate position of the ACE enzyme in a multistep pathway that can stabilize at different steady states depending on factors that influence different parts of the system. Niederberger et al have published extensively on this general topic [29], mainly in relationship to the net flux of substrates and products through the multistep pathways leading to the synthesis of essential metabolites such as arginine and tryptophan. From their theoretical treatment and observations in their experiments they concluded that increases of between 10-fold and 50-fold in the activities of four enzymes that are intermediate in the tryptophan synthetic pathway increased the total flux (that is, overall synthesis) of tryptophan by at most 30%, while a fourfold decrease in the activities of individual enzymes caused no more than a 25% decrease in total flux.

We approached the same type of problem in the renin-angiotensin system with the help of a generally applicable set of programs for simulating the behavior of complex interacting systems (STELLA®; High Performance Systems Inc., Hanover, NH, USA). Figure 4 shows the simplest model that yielded informative results with the endocrine arm of the renin-angiotensin system [30]. Figure 4 is essentially a diagrammatic formalization of relationships between the genes, proteins, enzyme substrates, and products of the system. The feedback loop between blood pressure and renin production is not included in this simplest model, nor do we include the role of ACE in the destruction of the kinins. However, later in this article we see that the conclusions drawn from the simulations are not affected by these simplifications. Table 1 lists some of the equations and constants that are required in modeling the dependence of the various components on each other. (A complete set is available from us on request.) For example, equation 2 describes how the change in concentration of an intermediate Q is determined by the rate of formation of Q from its precursor P minus the rate of conversion of Q to the product R and minus the rate of "clearance" of Q, where clearance means loss through the kidney, destruction by other enzymes, etc. Equation 6 describes the rate of conversion of angiotensin I (Ang I) to angiotensin II (Ang II) and assumes that this conversion is governed by Michaelis-Menten kinetics. Although precise values for many of the variables and constants are not known, their relative values can be estimated. For example, it is quite certain that the plasma concentration of Ang I (in the picomolar range) [31] is well below the K_m of ACE (which is in the micromolar range) [32], while the plasma concentration of AGT is in the same range as the K_m of renin [32–34].

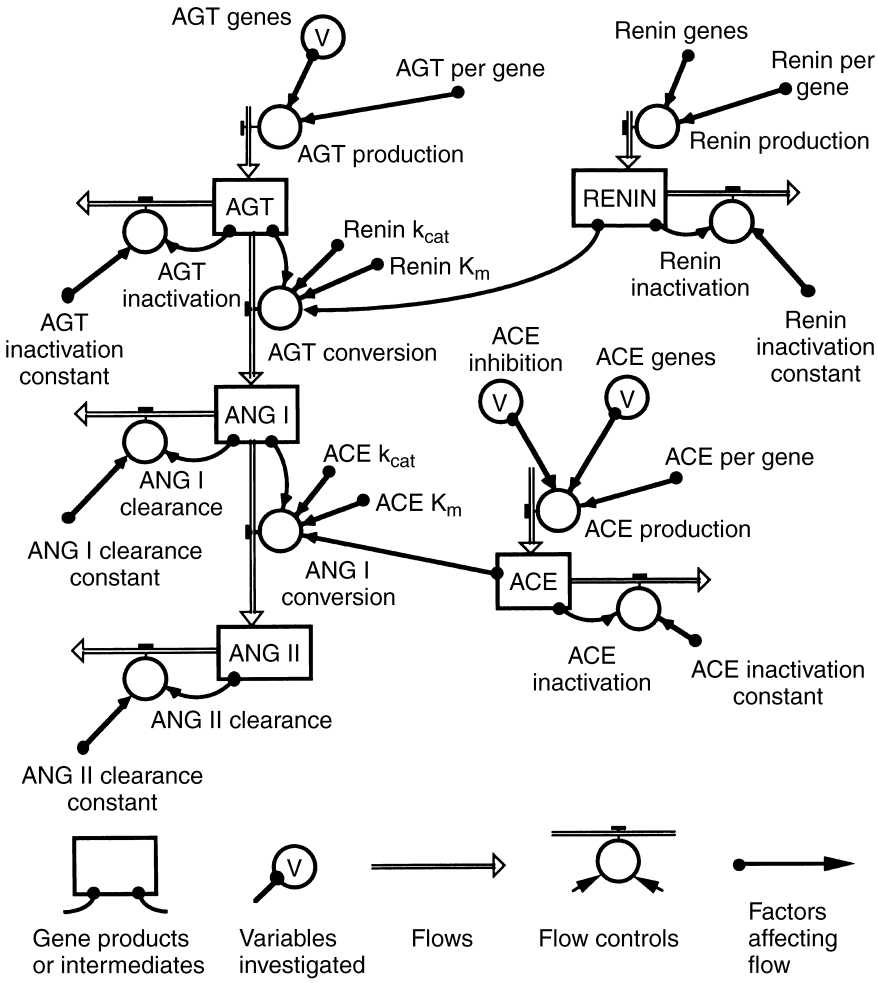

Fig. 4. The simplest representation of the endocrine arm of the renin-angiotensin system that was informative in the computer simulations with STELLA®.

Table 1. Chief equations and constants required for simulating the renin-angiotensin system

Equations
(1) Change of [Protein A][a] = (Number of A genes × Rate of producing A per gene) − (Inactivation rate of A)
(2) Change of [Intermediate Q] = (Rate of P to Q) − (Rate of Q to R) − (Clearance rate of Q)
(3) Inactivation rate of A = [Protein A] × Inactivation constant for A
(4) Clearance rate of Q = [Intermediate Q] × Clearance constant for Q
(5) AGT → Ang I = [AGT] × [Renin] × Renin kcat/([AGT] + Renin K_m)
(6) Ang I → Ang II = [Ang I] × [ACE] × ACE kcat/([Ang I] + ACE K_m)
Constants for which values must be chosen
K_m and kcat for renin and ACE
Inactivation constants for AGT, Renin and ACE
Clearance constants for Ang I and Ang II

[a] Square brackets = concentration

However, because precise values of many of the relevant constructs are not known, the numerical values resulting from our simulations should not be interpreted literally. Nevertheless, the relationships between the variables revealed by the simulations are robust because they are relatively insensitive to the exact values assigned to the constants.

We used the computer simulation to answer two questions: Why does a genetically determined threefold variation in AGT production alter blood pressure, while an exactly comparable change in ACE production does not? Why do ACE inhibitors affect blood pressure, while genetically halving ACE levels has essentially no effect?

Figure 5A and B illustrate simulations that help answer the first question. Figure 5A shows the simulated plasma levels of Ang I and Ang II when the number of *Agt* genes is varied from one through three, but the number of *Ace* genes is the normal, two. Figure 5B shows the simulated levels when the number of *Ace* genes is varied, but the number of *Agt* genes is kept constant at two. All parameters other than gene numbers are identi-

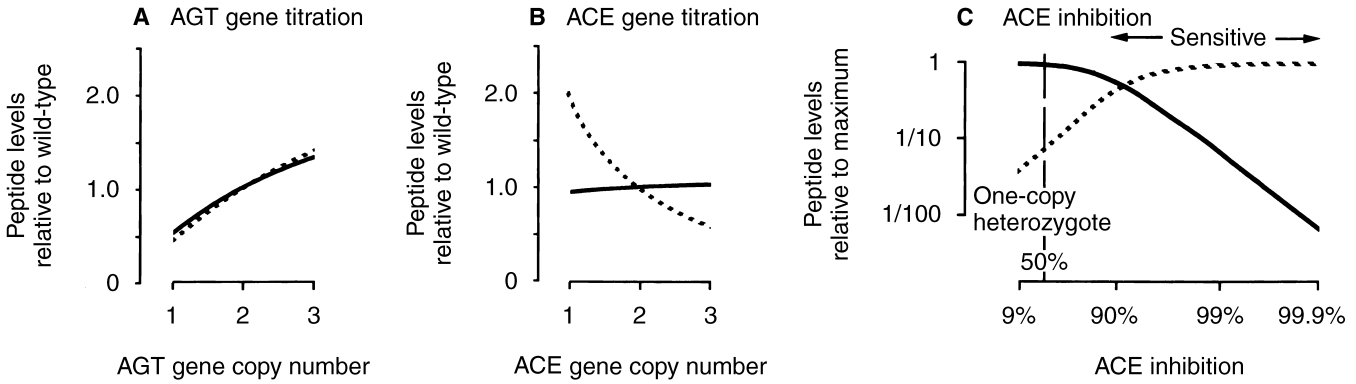

Fig. 5. Computer simulations of the angiotensin I (dotted line) and angiotensin II (solid line) concentrations when the following occurs: (*A*) the number of copies of the *AGT* gene is varied; (*B*) the number of copies of the *ACE* gene is varied; (*C*) the extent of inhibition of ACE with a converting enzyme inhibitor is varied. The scales in C are logarithmic. The arrows in C depict the region where Ang II levels are sensitive to further increases in the extent of ACE inhibition. Other details of the simulations are described in [24].

cal in these two simulations. The results are clear and in agreement with the genetic experiments: A progressive increase in *Agt* gene copy number yields a progressive increase in both Ang I and Ang II levels (the latter being equated to blood pressure in this simulation), while the same range of increase in *Ace* gene number has virtually no effect on Ang II levels, although it has a marked effect on Ang I.

The ability of the simulation to replicate the experimental observations is satisfying and a necessary prerequisite before any credence can be put on the procedure, but its chief value is in leading to a clearer understanding of the underlying relationships. In the present case, these relationships were most easily conceptualized by considering the one-copy animals in which either AGT or ACE is less than normal. When AGT decreases, the production of Ang I decreases, which, when ACE is constant, leads to a parallel decrease in Ang II and so in blood pressure. When ACE decreases, the conversion of Ang I to Ang II decreases, which, because Ang I production has not been changed, causes an increase in the steady-state plasma concentration of Ang I, which offsets the effects of the decrease in ACE activity so that Ang II production and blood pressure are virtually unaffected.

An answer to the second question—why ACE inhibitors reduce blood pressure when a genetically induced half normal level of ACE does not—required extending the simulation to consider more severe decreases in ACE activity. The results are most easily presented (Fig. 5C) as a plot of Ang I and Ang II levels against the inhibition of ACE, with the simulation using exactly the same parameters as in the two previous examples except that the decrease in ACE activity now covers a wider range (9% inhibition, 90%, 99%, etc.), and both axes of the graph are now logarithmic to accommodate this wide range. The result is again clear: At low extents of ACE inhibition (including the 50% level equivalent to the *Ace* one-copy mice), an increase in Ang I concentration offsets the decrease in ACE activity so that Ang II concentrations remain essentially constant. However, Ang I concentrations cannot increase indefinitely; they eventually plateau when "clearance" of Ang I by loss through the kidney and/or by degradative pathways account for the majority of its production. Once this Ang I plateau is reached, further decreases in ACE activity are no longer offset, and thus a decrease in Ang II, and thence of blood pressure, ensues. Note that the increases in Ang I concentration induced by the genetic changes in expression of either the *AGT* or the *ACE* gene (Fig. 5A, B) are up to about two times normal, while the increase induced by ACE inhibition is to about 20 times normal (the scales in Fig. 5C are logarithmic). The implication of this difference is that the genetically induced changes are insufficient to move the system into the region where ACE differences have an effect.

Space does not permit a detailed description of additional simulations that include the feedback induction of increases in renin production caused by decreases in blood pressure or by ACE inhibition and the effects of ACE on bradykinin concentrations. However, their bearing on the present topic can be summarized by noting that increased renin production induced by a decrease in blood pressure acts homeostatically to increase Ang I and Ang II concentrations, and so tends to restore blood pressures. In contrast, increased renin production induced by ACE inhibition has virtually no effect on Ang II concentrations; its effects are on Ang I concentrations. Other simulations that include bradykinin and its inactivation by ACE show that the plasma level of this peptide is inversely affected by changes in the level of ACE; these effects are not offset by secondary changes. The relative importance of Ang II and bradykinin in the control of blood pressure is not predictable by simulations of this type. However, the absence of a decrease in blood

pressure in mice with half normal levels of ACE suggests that its effects in this context are not important. Whether this is the case in humans is still a matter of debate. Thus, in humans, there is no consensus regarding the effects on blood pressure of the genetic variations in ACE levels associated with the *I/D* polymorphism [35]. The diversity in the human data may be caused by differences in the genetic backgrounds of the several study populations and/or by the effects of differences in dietary salt intake on the kallikrein-kinin arm of the system.

Three firm conclusions follow from the mouse genetic experiments and the accompanying simulations: first, modest genetically-induced changes in expression of the *Agt* gene directly and proportionately affect plasma Ang II concentrations and blood pressure. Second, similar changes in expression of the *Ace* gene do not affect Ang II concentrations or blood pressure because they are offset by unavoidable concomitant changes in Ang I concentrations, although bradykinin concentrations are affected by these changes in *Ace* gene expression, and third, the major effects of ACE inhibitors on Ang II concentrations (and hence blood pressure) do not commence until a threshold is reached beyond which changes in the concentration of Ang I no longer occur, with the result that further progressive inhibition of the enzyme by the drug is effective in changing the concentration of Ang II and blood pressure.

Experimental validation of the first conclusion with respect to the general effects of a half-normal expression of the *Agt* gene has been published. Both Ang I and Ang II plasma concentrations decrease by approximately 50% [19]. Our unpublished observations validate the reverse situation: increased expression of the *Agt* gene causes a comparable increase in the plasma levels of both peptides. The second conclusion is validated by our unpublished finding that in the *Ace* one-copy animals, Ang I concentrations in plasma are increased by almost 50%, but Ang II concentrations are unaffected. Bradykinin concentrations in the *Ace* one-copy animals are increased by 20%, as predicted qualitatively by the simulations. The validity of the third conclusion has already been very well documented by Campbell, Kladis, and Duncan in an extensive study in rats of the effects of a wide range of doses of the ACE inhibitor perindrol; the authors found that "*when Ang I levels were unable to increase further, higher perindrol doses caused plasma Ang II levels to fall*" [36].

THE NATRIURETIC PEPTIDE SYSTEM

The renin-angiotensin system is by no means unique with respect to the effects that genetically determined changes in quantitative gene expression have on blood pressure. For instance, the natriuretic peptide system has comparable effects, although in general it acts in the opposite direction. The natriuretic peptides, atrial natriuretic peptide and brain natriuretic peptide, are synthesized primarily in the heart, where their expression is stimulated by factors that stretch cardiac muscle [37]. Three receptors implement their downstream function, of which the one most directly related to blood pressure is the guanyl cyclase natriuretic peptide receptor A (NPRA), the product in the mouse of the gene *Npr1*. A gene titration experiment with the *Npr1* gene is illustrated in Figure 3E in which the linear relationship between gene copy number and gene expression is particularly well demonstrated [38]. Once more, the blood pressures of the resulting animals (Fig. 3F) are affected by quantitative differences in expression of the gene, although in this case, in the opposite sense from the *Agt* gene. *Npr1* is therefore another gene for which quantitative variations affect blood pressure.

An important caveat is necessary, however. Proof by mouse experiments that quantitative changes in expression of *Npr1* (or any other gene) affect blood pressure or its homeostasis make a strong argument that comparable changes in humans are also likely to influence blood pressure. However, the circle is not closed until variants of the candidate gene are proven to occur in human populations and are demonstrated experimentally to have similar effects on the expression of the gene or the function of its product. Such studies have not yet been done with the *NPRA* gene in humans.

HETEROZYGOTES

An important distinction exists between experiments to investigate the effects of quantitative changes in gene expression from those that investigate the effects of absence of gene function. The latter, which include conventional gene knockout experiments, are often more qualitative than quantitative. Nevertheless, the heterozygotes generated in the course of a conventional gene knockout experiment are, with a few exceptions such as when a gene is X-linked or is subject to imprinting, half of a quantitative gene titration experiment and, as such, can provide a wealth of quantitative information. Unfortunately, this information is largely untapped because only a small proportion of investigators study their heterozygotes in detail, for the obvious reasons that the homozygotes are more dramatic and the phenotype of the heterozygotes is often subtle and easily overlooked.

Failure to detect any change in blood pressure or any other phenotype of interest in heterozygous null animals should be accepted with caution when the homozygous null animals have a marked phenotype. In some cases, as with the *Ace* gene one-copy animals, the apparent absence of a heterozygous phenotype can lead to a better understanding of the overall system. Indications are that this may also apply to our recent work with the gene

Table 2. Effects on renal functions of *NKCC2* genotype

	NKCC2 genotype		
	+/+	+/−	−/−
NKCC2 protein *% wild-type*	100 ± 7	54 ± 8	0 ± 0[a]
Urine volume *mL/day/20 g body weight*	1.6 ± 0.2	1.6 ± 0.3	10.0 ± 0.9[a]
Urine osmolality *mOsm*	2300 ± 70	2350 ± 65	371 ± 20[a]
Plasma renin concentration *ng Ang I/mL/hr*	23.5 ± 4.5	23.5 ± 5.0	751 ± 76[a]

[a] $P < 0.0001$ versus +/+ and +/−

coding for the kidney-specific sodium potassium chloride cotransporter NKCC2. Loss of function mutations in this gene in humans [39], like CLCNKB, cause Bartter's syndrome. Mice homozygous for inactivation of the *NKCC2* gene exhibit severe polyuria and dehydration and die before weaning; a small proportion (~10%) of the homozygotes survive if treated with indomethacin shortly after birth, although the survivors have severe hydronephrosis [40]. Heterozygous mice, with one functional copy of the *NKCC2* gene, express only half of the normal amounts of the cotransporter in their kidneys. However, they have no detectable disturbances in their ability to concentrate urine or in their plasma renin concentrations, despite the fact that both of these features show extreme abnormalities in the homozygous null animals (Table 2).

Furosemide and bumetanide are two inhibitors of the NKCC2 cotransporter that are used in patients to control total body fluids and reduce blood pressure [41]. Administering furosemide to wild-type mice replicates most of the disturbances of the null homozygotes [40]. The situation is consequently very similar to the paradox encountered in the *Ace* gene experiments vis à vis the efficacy of ACE inhibitors, namely that a genetically induced 50% decrease in the expression of the gene has no obvious effects, but a drug that inhibits the gene product is nonetheless effective. Because the computer simulations of the renin-angiotensin system resolved the paradox with ACE, we are embarking on a similar endeavor to model ion transport and ion balances within and around a simplified representation of the cells in the thick ascending limb of the loop of Henle and in the macula densa where NKCC2 is expressed. Our aim is to determine why the decrease in NKCC2 to 50% has no effect, when the drug has such marked effects.

OVERALL STATUS

In the foregoing, we presented data demonstrating that modest changes in the expression of two genes (*Agt* and *Npr1*) affect blood pressure in mice, while changes in a third (*Ace*) do not. However, we have also seen that the absence of a direct effect of modest changes in the expression of a gene does not extrapolate to an absence of relevance to hypertension and its treatment, even though it decreases considerably the likelihood that such changes contribute to the etiology of essential hypertension. To help summarize the overall situation, we have therefore compiled in Table 3 a list of genes and related transgenes in which the effects on blood pressure in mice have been reported. The relevant data from all the publications that we have found are tabulated as the differences from wild type in the blood pressures of animals either having zero, one, or three copies of the normal wild-type gene or having a transgene. Wild-type pressures are presented but should be interpreted with caution, since they are more sensitive than the differences to variations in the procedure for measuring pressures. In some instances, homeostatic changes have been reported that tend to restore pressures to normal; the corresponding pressure differences are given a superscript *h.* Their significance may be greater than suggested by the numerical value of the difference. To help the reader identify genes relevant to the genetic control of basal blood pressures in mice and therefore to the genetic etiology of hypertension, the bold type in Table 3 highlights those genes for which modest changes in expression affect blood pressure. Underlined genes are those for which modest changes in expression do not affect blood pressure in mice. Genes neither bolded nor underlined have either not been tested quantitatively or the current data are inconclusive. Even with these relatively restrictive criteria for acceptance, Table 3 includes 10 genes that are strong candidates for being involved in the etiology of essential hypertension. A greater number merit further work before they can be excluded. The quantitative information that is readily accessible through the study of animals heterozygous for a gene knockout suggests an exhortation to those engaged in this type of work: "Treasure your heterozygotes."

SINGLE NUCLEOTIDE POLYMORPHISMS

Single nucleotide polymorphisms (SNPs) are the most common form of genetic variability in human populations, and they occur within both coding and noncoding regions of genes. SNPs leading to differences in coded amino acids are obvious candidates for altering protein function. SNPs in the noncoding regions close to coding sequences are candidates for altering gene expression. Determining which of them actually cause differences in gene function or gene expression will not be easy. One approach is to replace the equivalent endogenous mouse gene with each of the human allelic variants and then investigate the phenotypes of the "humanized" mice. This procedure has already been successful in proving that the two amino acid sequence differences that

Table 3. Genes tested for their effects on blood pressure in mice

Gene	Common name	0 copy ΔBP	1 copy ΔBP	Wild-type BP	3 copy ΔBP	Transgenic ΔBP	Reference
Adra3	adenosin A3 R	−1		117			[44]
Ace		−33	−5	116			[28]
			+1[h]	117	−2		[27]
Adm	adrenomedullin		−4	112	+2		KMC
Adora2a	adenosinA2aR	+20		117			[45]
Adra1b	α1b adrenergic R	0		119			[46]
Adra2a	α2a adrenergic R	+3		128			[47]
Adra2b	α2b adrenergic R	0		118			[48]
Adra2c	α2c adrenergic R	0		138			[48]
Adrb2	β2 adrenergic R	0		114			[49]
Agt		−16[h]	−8[h]	130	+8		[20, 23]
Agtr1a	AT1aR	−22[h]	−10	108			[50]
		−43[h]	−17[h]	133			[51, 52]HSK
Agtr1b	AT1bR	−1		101			[53]
		−9		123			[54]
Agtr2	AT2R	+17	102				[55]
		+4	122				[56]
Ann6	annexin	0		105			[57]
Apoe	apolipoprotein E	+16		110			[58]
Arrb1	βarrestin 1	+4		114			[59]
Avp	vasopressin			122		−22	[60]
Bdkrb2	bradykininB2R	+15	+2	109			[61]
		+34(HS)	+14(HS)	115(HS)			[61]
		+7	+1	114			[62]
		+21	+19	115			[63]
		+4		70			[64]
		+16(HS)		75(HS)			[64]
				97		−20	[65]
Brs3	bombesin R3	+1	110(4m)				[66]
		+15	116(10m)				[66]
Cgrpa	calcitonin gene-related peptide	+5		129			[67]
		+21		118			[68]
Cnr1	cannabinoid R	+10		113			[69]
Crhr2	CRHR	+10		88			[70]
Drd1	dopamineD1R	+20	+23	84			[71]
Drd3	dopamineD3R	+23	+20	97			[72]
Edn1	ET1		+11	105			[73]
				88		−2	[74]
Ednra	ETAR		+5	125			[75]
Ednrb	ETBR	+15(−/s)	−2(+/s)	110			[75]
Fgf2	FGF2	−21	0	99			[76]
Gh	growth hormone			88		0	[77]
Glut4			+36	102			[78]
Hsd11b2	11βHSD2	+25[h]	−7	121			[79]
Irs1		+11		99			[80]
Klk	kallikrein			101		−22	[81]
Klkbp	kallikrein binding protein			101		−12	[82]
Ldlr	LDL R	+43		111			[83]
Mme	NEP	−23		121			[84]
Nos1	nNOS	−5		95			[85]
Nos2	iNOS	−2	−1	116			[86]
Nos3	eNOS	+20		97			[87]
		+18	+4	122			[86]
				99		−18	[88]
Nppa	ANP	+22	−2	134			[89]
			+27(HS)	118(HS)			[89]
		+22(HS)		113(HS)			[90]
				104		−28	[91]
Nppb	BNP			125		−25	[92]
		−3	−5	121			[93]
		−1(HS)	−2(HS)	118(HS)			[93]
Npr1	GC-A	+20	+8	77			[94]
	NPRA	+16		136			[95]
			+9	123	−5		[38]
Npr3	NPRC	−9	−1	119			[96]

(*continued*)

Table 3. (Continued)

Gene	Common name	0 copy ΔBP	1 copy ΔBP	Wild-type BP	3 copy ΔBP	Transgenic ΔBP	Reference
Npy2r	NPY Y2R	−11		117			[97]
Lep	Ob	−14		106			[98]
Ptgerep1	EP1	0		98			[99]
Ptgerep2	EP2	+6		101			[100]
		+28(HS)		105(HS)			[100]
		−13		122			[101]
		−5(HS)		122(HS)			[101]
Ptgerep3	EP3	−1		120			[99]
Ptgerep4	EP4	+7		101			[99]
Ptgir	PGI2R	−3		85			[102]
Ptgs1	COX1	+2	0	122			SGM
		−8(LS)		101(LS)			TMC
Pthrp1	PTHrP			135		−14	[103]
Pth1r	PTH/PTHrPR			133		−16	[104]
Ren 1c		−28	−3	86			[105]
Ren 1d		−2(M)	−1(M)	94(M)			[106]
		−13(F)	−8(F)	94(F)			[106]
Ren 2		−1	+1	85			[107]
Scnn1b	βENaC	+8		130			[108]
		−8(LS)		130(LS)			[108]
Serca3	SR Ca ATPase 3	+1		78			[109]
Slc12a1	NKCC2/BSC1		+1	114			NT
Slc12a2	NKCC1/BSC2	−23	−14	92			[110]
		−8		108			[111]
Slc12a3	TSC	-2^{h}		87			[112]
		−14(LS)		86(LS)			[112]
Slc9a1	NHE1			99(HS)		+21(HS)	[113]
Slc9a3	NHE3	−9	+2	92			[114]
Tbxa2r	TXR	+7		93			[115]

Bold italics indicates ***genes*** for which quantitative differences in expression have been demonstrated to affect blood pressure. Underlined italics indicates *genes* for which quantitative differences in expression do not affect blood pressure. Italics (not bold or underlined) indicate *genes* for which quantitative differences in expression have not been made or are inconclusive. Methods of BP measurements differ in various experiments. As much as possible, the listed values are mean BP. Where investigators report more than one set of BP measurements, we have selected one that appears most representative. Where a difference between sexes has been reported, the sexes are distinguished. Homeostatic adjustments have been demonstrated where indicated by a superscript *h*. Personal communications are from: KMC, Kathleen M. Caron; TMC, Thomas M. Coffman; HSK, Hyung-Suk Kim; SGM, Scott M. Morham; NT, Nobuyuki Takahashi. Abbreviations are: F, female; HS, high salt; LS, low salt; m, month; M, male; S, piebald mutation.

characterize three common alleles of the human apolipoprotein E gene result in readily distinguishable effects on atherogenesis in mice [42]. Analogous experiments with human allelic variants, coding or noncoding, that may be found in genes demonstrated to influence blood pressure or its homeostasis in mice should be equally informative, although care will be needed in instances in which the protein under test interacts with other proteins or receptors that have species-specific properties.

COMBINATIONS OF VARIANTS

A formidable task still remains for experimental geneticists who study mice and humans: to test and sort out the effects on blood pressure of combinations of genetic variations both within systems (such as two variants within the renin-angiotensin system) and between systems (such as one variant in the renin-angiotensin system combined with another variant in the natriuretic peptide system). The chief difficulty facing this type of investigation in mice is the need to ensure a constant genetic background in the animals involved [43]. Backcrossing to a constant inbred background before making the combinations is one possible approach. Combining mutants that were generated and maintained within a single already inbred strain is another possibility. Answering the question of whether small variations in different genes have simple additive effects on blood pressure must await completion of these experiments.

CONCLUDING REMARKS

This article has developed the thesis that quantitative differences in the expression of many genes can affect blood pressure in mice and, by inference, in humans with all of their normal homeostatic processes intact. Several implications of this thesis merit comment with respect to the present status and future prospects of diagnosing and treating essential hypertension. The chief tool for diagnosis is still the sphygmomanometer, which in a very real sense only estimates the difference between those genetic and environmental factors that tend to increase

blood pressure and those that tend to decrease it. Because physicians currently have no means of uncovering the discrete factors underlying essential hypertension, determination of the most suitable drugs and doses for each patient is largely empiric. If the starting hypothesis of this perspectives has any validity, and the mouse experiments suggest that it does, then the future can be viewed optimistically. Once a tally, even if incomplete, has been made of the genes whose expression affects blood pressure, a new generation of diagnostic tools can be designed. Basic scientific and clinical data should eventually tell us which of the alleles of the tallied genes are potentially protective and which are damaging. This information should enable the design of DNA microchips of relatively modest complexity to determine which alleles are present in a given patient. The net result can be the selection of treatments tailored to the specific individual, thereby exposing fewer persons to side-effects from treatments that are less than optimal.

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